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Antibody Response from Whole-Cell Pertussis Vaccine Immunized Brazilian Children against Different Strains of *Bordetella pertussis*

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Abstract. *Bordetella pertussis* is a gram-negative bacillus that causes the highly contagious disease known as pertussis or whooping cough. Antibody response in children may vary depending on the vaccination schedule and the product used. In this study, we have analyzed the antibody response of cellular pertussis vaccinated children against *B. pertussis* strains and their virulence factors, such as pertussis toxin, pertactin, and filamentous hemagglutinin. After the completion of the immunization process, according to the Brazilian vaccination program, children serum samples were collected at different periods of time, and tested for the presence of specific antibodies and antigenic cross-reactivity. Results obtained show that children immunized with three doses of the Brazilian whole-cell pertussis vaccine present high levels of serum antibodies capable of recognizing the majority of the components present in vaccinal and non-vaccinal *B. pertussis* strains and their virulence factors for at least 2 years after the completion of the immunization procedure.

INTRODUCTION

Bordetella pertussis is a gram-negative bacillus that causes the highly contagious disease known as pertussis or whooping cough, which is responsible for a worldwide estimated 50 million illness cases (90% of which are in developing countries) and 400,000 deaths each year.[1,2] It remains an important cause of disease in both very young infants and in adolescent and adult populations despite the routine use of vaccination programs.[2,3] In fact, *B. pertussis* seems to be re-emerging, featuring a modified epidemiology with a high incidence in early infancy and rising incidences in older children and adults.[4,5] Several explanations have been put forward for the resurgence of *B. pertussis* infection in vaccinated populations.[6] An apparent increase in *B. pertussis* incidence may result from improved surveillance, changes in case definition, and better diagnostic techniques.[7] However, it seems unlikely that these factors solely explain all cases in which a dramatic rise in *B. pertussis* has been observed. Other factors that might affect the incidence of *B. pertussis* include demographic changes, waning vaccine-induced immunity, changes in vaccine quality and/or vaccine coverage, or a decrease in the vaccine efficacy due to antigenic differences between circulating isolates and vaccinal strains.[8] These antigenic differences may arise from genetic selective pressure induced by long term vaccination, or through changes in vaccine manufacturing that affect the antigens expressed by the vaccinal strains.[7,8]

Several vaccine combinations have been used to improve the establishment of the immunization programs, to consolidate the use of polyvalent vaccines, and to increase the coverage of each vaccine.[7] Acellular pertussis vaccines were proven to be efficient and safe, due to the lower reactogenicity; in several countries their use has been preferred in the vaccination instead of the whole-cell pertussis.[9–11] Brazil has been producing diphtheria-tetanus-pertussis vaccine since 1953, and from 1980, the 137 *Bordetella pertussis* strain, from the National Institutes of Health (Bethesda, MD), was chosen to be used as antigen for the production of the whole cell pertussis vaccine (DTwP).[12]

Recent studies indicate that both immunization and infection during childhood do not lead to a permanent immunity against *Bordetella pertussis* and, as a consequence, older children and adults are the main reservoirs of the infection.[4,13] The aim of this study was to evaluate the antibody response of the whole cell pertussis vaccine immunized Brazilian children to various *B. pertussis* strains and their virulence factors in different periods of time after completion of the immunization process.

MATERIALS AND METHODS

Chemicals and reagents. Tween 20, bovine serum albumin (BSA), goat anti-human alkaline phosphatase (IgG-AP), and p-Nitrophenyl Phosphate (pNPP) were purchased from Sigma (St. Louis, MO). BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and nitroblue tetrazolium (NBT) were from Promega Corp. (Madison, WI). Purified pertussis toxin (PT), pertactin (PRN), and filamentous hemagglutinin (FHA) were kindly provided by Dr. Rino Rapuolli (CHIRON S.p.A Laboratory, Siena, Italy).

Strains and growth conditions. The *Bordetella pertussis* strains used in the experiments were: 21A1, isolated from nasopharyngeal aspirate from an infant hospitalized with whooping cough clinical symptoms;[14] 137, obtained from the National Institutes of Health (NIH) and used for the preparation of the whole-cell vaccine in Brazil; 143, obtained from NIH; and Tohama, the Japanese vaccine strain. Bacteria were grown at 35.5°C for 24 hours on Bordet–Gengou agar plates[15] supplemented with defibrinated sheep blood at 25% and subcultured in Stainer and Scholte medium.[16] For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis, bacteria grown on Bordet–Gengou agar plates at 10×10^9/mL were centrifuged at $7{,}000 \times g$ for 45 minutes at 4°C and cell pellets washed three times in saline solution. Samples were prepared in Laemmli buffer and boiled for 10 minutes.

Human serum samples. Children ($N = 96$) from Rio Preto, São Paulo, Brazil, were immunized with three doses of the whole-cell diphtheria-tetanus-pertussis vaccine, produced by Butantan Institute (São Paulo, Brazil), using *Bordetella pertussis* strain 137 from NIH as immunogen. Serum samples

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were collected after 2 (N = 12), 4 (N = 13), 6 (N = 22), 12 (N = 26), and 24 (N = 23) months after the third dose, the time when the children were aging from 6 months to 3 years. The samples were centrifuged and the sera separated and stored at –20°C. Control serum samples were obtained from non-immunized children aging from 6 months to 1-year-old (N = 8). This protocol was approved by the local ethics committee and the parents of the involved children authorized their participation in the study.

Enzyme linked immunosorbent assay (ELISA). Microtiter plates were coated with 100 µL of *B. pertussis* cell extracts or purified pertussis toxin (PTx), PRN, and FHA (10 µg/mL; overnight at 4°C). Plates were blocked with 5% BSA in phosphate buffered saline (PBS) (10 mM sodium phosphate containing 150 mM NaCl, pH 7.2) for 4 hours at room temperature, and then incubated with serum samples in PBS/5% BSA. After 1 hour, plates were washed with PBS/0.05% Tween 20 and incubated for 1 hour at room temperature with goat anti-human IgG-AP diluted 1/1000. Plates were washed and the reactions developed with pNPP substrate according to the instructions of the manufacturer (Sigma).

Electrophoresis and western blot. Samples of the different *B. pertussis* strains cell pellets and toxins (PTx, PRN, and FHA) were solubilized in non-reducing sample buffer and run on SDS-PAGE.[17] Gels were stained with Coomassie blue or blotted onto nitrocellulose.[18] After transfer, the membranes were blocked with PBS containing 5% BSA and incubated with human sera samples (diluted 1/1000) for 1 hour at room temperature. Membranes were washed three times with PBS/0.05% Tween 20 for 10 minutes and incubated with goat anti-human IgG-AP (diluted 1/3000) in PBS/1% BSA for 1 hour at room temperature. After washing three times with PBS/0.05% Tween 20 for 10 minutes, blots were developed using NBT/BCIP according to the manufacturer's instructions (Promega).

Statistical analysis. The Student's t test was used to determine the significance of the differences between the mean values of the groups. The minimal level of significance was considered as $P < 0.05$.

RESULTS AND DISCUSSION

Infection by *B. pertussis* is still an important cause of morbidity and mortality among children in many parts of the world.[1,2] It is also being recognized as an increasingly significant agent of respiratory disease in adults.[19] The results of recent trials have indicated that the efficacy of the whole-cell vaccine is variable but also showed that the efficacy of acellular vaccines is similarly variable.[20,21] Children are considered to be adequately immunized when they received at least the first three doses of the vaccine.[20] In Brazil, it is recommended that the whole-cell pertussis vaccine should be administered at 2, 3, and 4 months of age, with a reinforcement dose at 15 months and at 6 years of age. Vaccine coverage in 2000 and 2001 was approximately 90%, and during the same period, the number of annual cases did not exceed 2,000, keeping the coefficient of incidence around 1/100,000 habitants.[22]

In the present study, the presence of IgG antibodies anti-*Bordetella pertussis* was analyzed in serum samples of immunized children. Sera were tested against extracts of the vaccinal strains 137, 143, Tohama, and also against 21A1, isolated from a patient presenting whooping cough in São Paulo, SP, Brazil. The latter strain, when used for the preparation of a whole cell experimental pertussis vaccine, showed to be the most antigenic and protective in the Kendrick test when compared with other vaccinal strains.[23] The analyses, conducted here, were performed to evaluate whether the serum of children vaccinated with the 137 NIH strain would recognize efficiently the antigens of other *B. pertussis* vaccinal strains, as well as the one circulating in Brazil.

Serum samples from male and female children immunized with three doses of the whole-cell pertussis vaccine, and aging from 6 months to 3 years, were analyzed by ELISA for the presence of specific IgG antibodies. Serum samples were collected 2, 4, 6, 12, and 24 months after the third vaccine dose. Figure 1 shows that 2 months after the third dose of the whole-cell pertussis vaccine, children presented high levels of specific antibodies in their sera, which remained stable for at least 2 years. Moreover, no significant differences were observed in the antibody response values against other *B. pertussis* strains, indicating that this vaccine induces a good repertoire of specific antibodies able to recognize antigens present in vaccinal and non-vaccinal strains.

These sera were grouped according to the age of the children, i.e., from 6 to 12, 13 to 24, 25 to 36 months, corresponding to 36%, 34%, and 22% of the total number of samples, respectively, and also evaluated by ELISA. Control sera, from nonimmunized children, aging from 6 to 12 months, corresponding to 8% of the samples, were included in the analysis. Figure 2 shows that the antibody response to the vaccine and to other *B. pertussis* strains was similar in all groups, indicating that child, since the first year of life, is already ready to fully respond to the whole-cell pertussis vaccine.

The reactivity of pools of sera, prepared with samples collected 2, 4, 6, and 12 months after the third dose of the vaccine, was also evaluated by ELISA against the *Bordetella* toxins PT, FHA, and PRN. Figure 3 shows that high levels of specific antibodies to *B. pertussis* virulence factors are present, at least, until 12 months after the completion of the immunization process. For PT and FHA, the antibody response kinetics were similar; for PRN, a specific antibody production peak after 4 months, which was kept at 6 months and decreased at 12 months was observed, after conclusion of the immunization schedule.

A pool of children sera, corresponding to samples collected 4 months after the third dose of the vaccine, was tested by western blotting for cross-reactivities, using the *B. pertussis* extracts from 137, 143, Tohama, and 21A1 strains, and the antigens PTx, PRN, and FHA. Figure 4 reveals that this serum pool was equally capable of strongly recognizing components present in the four *B. pertussis* strains, as well as the purified virulence factors.

Several proteins were identified in the vaccine strain 137, as well as in the other studied strains, however, data in the literature are controversial in relation to which are the major antigens involved in the immunity and clinical protection. Antibodies against PT and PRN were positively correlated with clinical protection against whooping cough in humans.[23,24] The adhesion PRN, present on the surface of *B. pertussis,* seems to be a component of major importance for both T-cell and antibody-mediated immunities to *B. pertussis*.[25] On the other hand, FHA has been reported as a major adhesion component of *B. pertussis*, involved in the interaction of the bacteria with macrophages.[25] Therefore, IgG antibodies against the

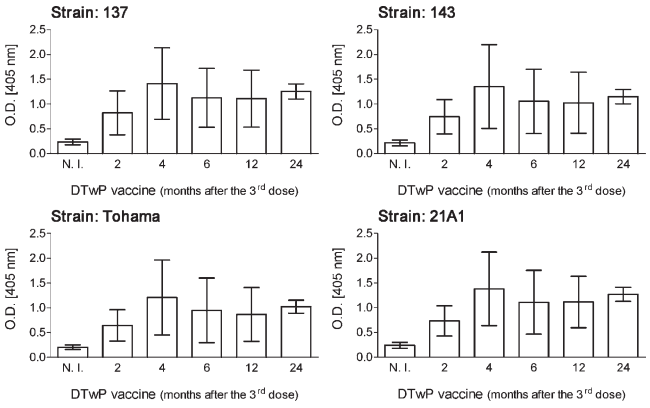

FIGURE 1. Serum antibody response against extracts of *B. pertussis* strains. ELISA plates were coated with 1 μg of the extracts of each of the four *B. pertussis* strains (137, 143, Tohama, and 21A1) and incubated with the serum samples ($N = 96$), diluted 1:100, obtained from children after 2, 4, 6, 12, and 24 months after the completion of the immunization with the whole-cell pertussis vaccine. As control, sera from nonimmunized (NI) children ($N = 8$) were used. After 1 hour of incubation, goat anti-human IgG-AP was added and the reaction developed with pNPP substrate. The absorbance of the samples was determined at 405 nm. The data presented correspond to the mean OD_{405} value ± SD of experiments carried out in duplicate.

B. pertussis virulence factors, present in the sera of immunized children, as here demonstrated, may be important in the control of whooping cough and the prevention of its dissemination in pre-school children.

In this study the cellular immune response to the whole cell *B. pertussis* vaccine was not determined but it is reasonable to

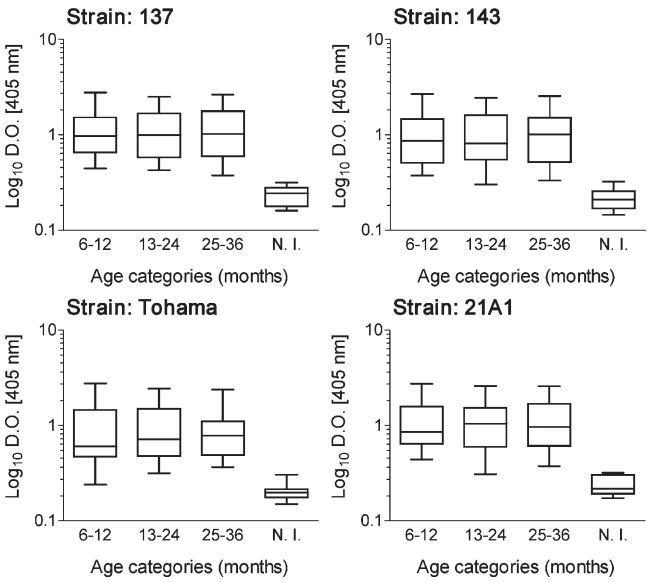

FIGURE 2. Influence of the children age in serum antibody levels against *B. pertussis* strains. ELISA plates were coated with 1 μg of the extracts of the four *B. pertussis* strains (137, 143, Tohama, and 21A1) and incubated with sera from immunized children (diluted 1:100), that were grouped according to their age, i.e., 6-12, 13-24, and 25–36 months, corresponding to 36%, 34%, and 22% of the total sample, respectively. As control, sera of nonimmunized (NI) children, aging from 6 to 12 months old ($N = 8$) were used. After 1 hour of incubation, goat anti-human IgG-AP was added and the reaction developed with pNPP substrate. The absorbance of the samples was determined at 405 nm. The data presented correspond to the mean OD_{405} value ± SD of experiments carried out in duplicate.

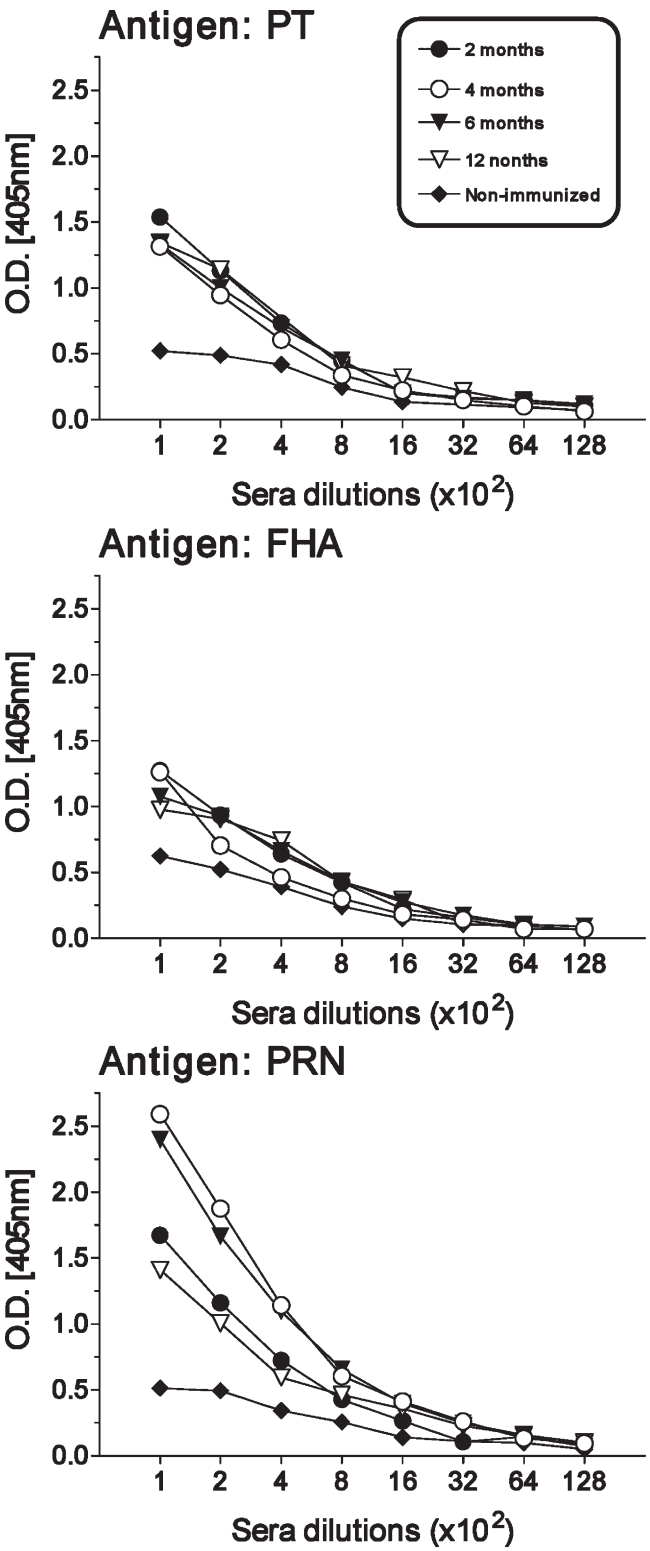

FIGURE 3. Serum antibody response against the virulence factors of *B. pertussis*. ELISA plates were coated with 1 μg of the *B. pertussis* antigens PT, PRN, and FHA, and incubated with increase dilutions of pools of sera, obtained from children after 2, 4, 6, and 12 months after the immunization with whole-cell pertussis vaccine. As control, a pool of sera from nonimmunized (NI) children was used. After 1 hour of incubation, goat anti-human IgG-AP was added and the reaction developed with pNPP substrate. The absorbance of the samples was determined at 405 nm. The data presented correspond to the mean OD_{405} value of experiments carried out in duplicate.

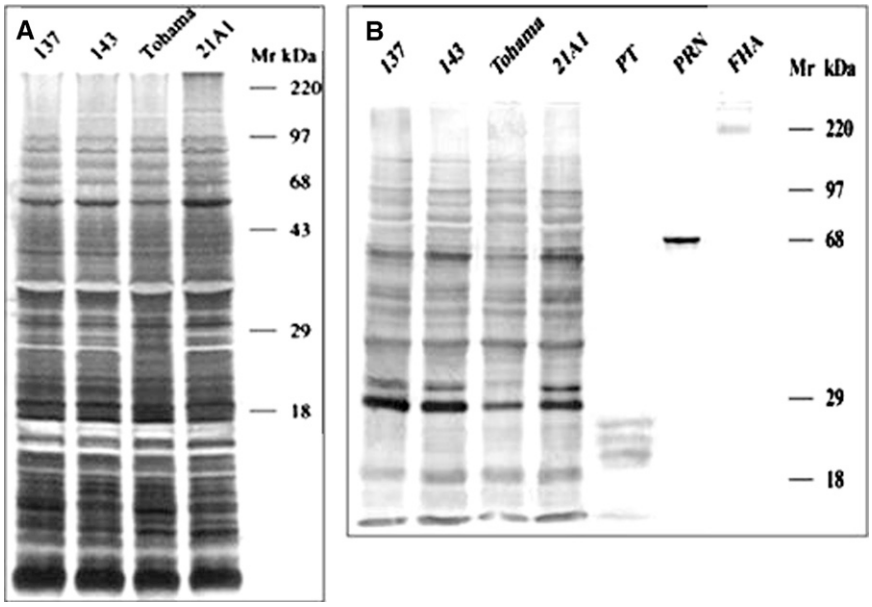

FIGURE 4. Cross-reactivity of the sera against toxins and extracts of *B. pertussis*. Samples of cell extracts from *B. pertussis* strains 137, 143, Tohama, and 21A1 and of the purified bacterial antigens PT, PRN, and FHA solubilized in non-reducing condition, were analyzed in a 7.5–20% gradient SDS-PAGE gels and stained by Coomassie blue **A** or electrotransferred to nitrocellulose membranes **B** and incubated with a pool of children sera (diluted 1:1000) obtained after 4 months of immunization with the whole-cell pertussis vaccine, followed by goat anti-horse/IgG-AP. The reaction was revealed with NBT and BCIP.

hypothesize that, because the pleiotropic effect of the polygenes regulating the quantitative antibody responsiveness to this complex immunogen, there is a general commitment of immunobiological factors influencing the effective immune response to this vaccine.[26] Moreover, it must be stressed that an efficacious vaccine must be proficient in induction of effective memory to relevant epitopes. These results clearly demonstrate that the entire cell vaccine provides specific neutralizing antibodies for a long period evidencing the participation of the main B and T cell protagonists of the acquired immune response to *B. pertussis*.

In conclusion, the data presented show that children immunized with three doses of the Brazilian whole cell pertussis vaccine possess high serum antibody levels against the majority of the components present in vaccinal and non-vaccinal *B. pertussis* strains, including the main virulence factors, for at least 2 years after the completion of the immunization. Although, some quantitative variations have been observed concerning the strain recognition, the results clearly demonstrate that this vaccine induces an effective immunologic memory.

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